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Strict control of transgene expression in a mouse model for sensitive biological applications based on RMCE compatible ES cells

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ABSTRACT

Recombinant mouse strains that harbor tightly controlled transgene expression proved to be indispensible tools to elucidate gene function. Different strategies have been employed to achieve controlled induction of the transgene. However, many models are accompanied by a considerable level of basal expression in the non-induced state. Thereby, applications that request tight control of transgene expression, such as the expression of toxic genes and the investigation of immune response to neo antigens are excluded. We developed a new Cre/*loxP*-based strategy to achieve strict control of transgene expression. This strategy was combined with RMCE (recombinase mediated cassette exchange) that facilitates the targeting of genes into a tagged site in ES cells. The tightness of regulation was confirmed using luciferase as a reporter. The transgene was induced upon breeding these mice to effector animals harboring either the ubiquitous (ROSA26) or liver-specific (Albumin) expression of CreER^{T2}, and subsequent feeding with Tamoxifen. Making use of RMCE, luciferase was replaced by Ovalbumin antigen. Mice generated from these ES cells were mated with mice expressing liver-specific CreER^{T2}. The transgenic mice were examined for the establishment of an immune response. They were fully competent to establish an immune response upon hepatocyte specific OVA antigen expression as indicated by a massive liver damage upon Tamoxifen treatment and did not show OVA tolerance. Together, this proves that this strategy supports strict control of transgenes that is even compatible with highly sensitive biological readouts.

INTRODUCTION

The ability to switch genes 'on' or 'off' in a particular tissue in the mouse at any defined time point is a powerful tool to investigate mammalian gene function in development, disease and various physiological processes. Currently, the regulated expression of transgenes has been achieved by two different methods, i.e. reversible transcriptional control employing regulated promoters and irreversible genetic control by the use of site-specific recombinases [reviewed in (1–5)]. Transcriptional systems have been established, in particular, employing the Tetracycline (tet) system to control transgene expression in mammalian cell culture (6–8) as well as in mice (9–11). According to the design of the expression modules, both gradual expression and stochastic, i.e. bimodal expression can be achieved (12).

A different mode of regulation is provided by genetic switches based on recombinases such as Cre or Flp. Most commonly for conditional gene activation, the specific gene to be switched on is usually separated from the promoter by a 'STOP' cassette that prevents the

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transcription and translation of the target gene. This 'STOP' cassette is in turn flanked by directly oriented *loxP* sites (13–18). The 'STOP' cassette usually comprises of single or multiple polyadenylation signal(s). Upon excision of the STOP cassette via Cre, the target gene is activated. An efficient method to achieve temporal regulation of Cre-mediated recombination is by fusing the Cre ORF to the mutated ligand-binding domain (LBD) of the human estrogen receptor (CreER^T). Currently, several steroid regulated forms of CreER^T recombinases are available (19–23) that can be activated by the synthetic ligand Tamoxifen (Tam).

While a plethora of transgenic systems for various biological applications could be generated, questions that require extremely tight control of transgenes in transgenic mice were not yet addressed. In particular, immune activation studies have been shown to be compromised by 'leaky', unregulated gene expression as e.g. revealed by successful DNA immunization even with non-induced expression cassettes (24). In transgenic mice, unintended basal antigen expression during embryonic development would result in tolerance since the antigen will be recognized as an endogenous ('self') antigen. Accordingly, even though disease models do exist that employ the above mentioned controlled tissue-specific gene regulation systems (25,26), their application toward immune activation studies was not yet evaluated.

We developed a mouse model that shows strict regulation of any transgene. We employed Cre-mediated inversion of the transgene rendering it under the control of the ubiquitously active ROSA26 promoter. Breeding with mice providing ubiquitously expressed or liver-specifically expressed CreER^{T2} allows activation of antigens by Tam at any time. The transgene cassette was introduced into the ROSA26 locus in a way that supports its exchange by recombinase mediated cassette exchange (RMCE), thereby providing a highly flexible approach for inducible transgene expression of choice. Here, we report the results from two transgenes, luciferase and Ovalbumin, and evaluate the strategy as a model for induced hepatitis.

MATERIALS AND METHODS

ROSA26 tagging plasmid and RMCE exchange vector construction

To create a platform ES cell line for RMCE-based ROSA26 targeting, we established a tagging vector based on pROSA26-1 (17) harboring the homology arms of the ROSA26 locus and additionally comprising the following components (i) adenoviral splice acceptor site (SA); (ii) non-interacting *FRT* sites (wild-type *FRT*, shown as F in the figure and the mutant *F5* site (27), respectively, flanking; (iii) an inverted luciferase (LUC) cassette flanked by inverted *loxP* sites followed by a puromycin *N*-acetyltransferase (PAC) gene; and (iv) a promoter and start-codon deficient neomycin phosphotransferase gene (Δ Neo). The design of the cassette upon homologous recombination in the ROSA26 locus is depicted in Figure 1.

The RMCE exchange vectors are based on pEMTAR (28) and harbor a multiple cloning site followed by the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) element with a translational ATG start codon positioned in frame with the non-interacting *FRT* and the mutant *F5* site, respectively.

In all ES cells evaluated in this study, the transgene of interest (Cre-activatable Ovalbumin or luciferase gene) was integrated in antisense and flanked by oppositely oriented wild-type *loxP* sites. The OVA coding sequence gives rise to a fusion protein comprising of three components (i) hsp 73-capturing N-terminal viral J domain of SV40 T-Ag (cT$_{77}$); (ii) 108-residue of the Ovalbumin fragment (aa 246–353 with a isoleucine to valine change at position 258) with well-characterized K^b- and A$^{d/b}$-binding epitopes (specifically recognized by OT-I or OT-II/D011 TCR) and (iii) the eGFP reporter (Schirmbeck, R., unpublished data).

Vector sequences and maps are available on request.

Cell culture

IB10 murine embryonic stem cells (mES) cells (subclone of E14 ES cell line) (29) were cultured on feeder cells [mitotically inactivated murine embryonic fibroblasts (MEF)] and maintained in DMEM+GlutaMAX-I (Gibco) supplemented with 15% fetal calf serum (heat inactivated: 30min at 56°C), penicillin (10 U/ml), streptomycin (100 μg/ml), 1 mM non-essential amino acids (Gibco), 1mM sodium–pyruvate (Gibco), 0.1 mM β-mercaptoethanol and in the presence of leukemia inhibitory factor (LIF). The cells were kept at 37°C and 7% CO_2 in humidifying incubators.

In vitro differentiation of ROSALUC mES cells. A total of 1×10^6 ROSALUC mES cells were seeded in 15ml DMEM medium (Gibco) supplemented with 10% fetal calf serum, penicillin (10 U/ml), streptomycin (100 μg/ml), 2mM L-glutamine in bacterial dishes. Suspension culture in bacterial dishes, in the absence of feeders and LIF for 5–7 days led to the formation of embryoid bodies. Embryoid bodies were centrifuged (500 rpm, 5 min) and plated on gelatinized 10 cm cell-culture dishes so that they could adhere and form outgrowths of differentiated cells. After 4–5 days, the cells were dissociated by trypsin EDTA (TEP) (Sigma) and split on to gelatinized six wells. Accordingly, samples were then harvested for subsequent analysis of luciferase activity.

Modification of mES cells

Homologous recombination of ROSA26 locus with the tagging vector. A total of 4×10^6 mES cells were harvested with TEP, centrifuged (1000 rpm, 5 min) and the cell pellet was washed once with PBS to remove any residual culture medium. For electroporation with the Gene Pulser (Biorad) cells were re-suspended in 1 ml Phosphate Buffered Saline (PBS) and 10 μg of the purified, XhoI linearized plasmid DNA was added. Electroporation was performed at 240 V and 475 μF capacitance (time constant = 10.2). After electroporation, the cell suspension was transferred to pre-warmed culture medium, seeded onto feeder coated 10cm cell-culture dishes and allowed to recover. After 48 h, puromycin was added at a concentration of 1 μg/ml to select the cells.

RMCE. All the targeting experiments in ROSALUC mES cells based on Flp mediated cassette exchange were performed using Lipofectamine™ 2000 (Invitrogen). For this purpose, 80–90% confluent ROSALUC mES cells seeded on gelatinized six-well dishes were co-transfected with the circular exchange vector and the Flp recombinase expression vector pFlpe (30) (usually at a DNA concentration ratio of 1:3 or 1:1 respectively) along with 10 μl of the Lipofectamine™ 2000 reagent as per the manufacturer's instructions. After 48 h the transfected ES cells were transferred to feeder coated 10 cm cell-culture dishes and selection pressure with G418 at a concentration of 0.4 mg/ml was applied. As a negative control, ROSALUC mES cells transfected with only the Flp recombinase expression plasmid was always included. Selection was usually carried out for 8–10 days during which it was ascertained that all the cells in the negative control were killed. Putative RMCE targeted G418 resistant subclones obtained were then picked and cultured in medium containing G418.

Stable transfection of ROSALUC mES cells with Cre expression vector. ROSALUC mES cells were stably transfected with a Cre recombinase expression vector, pPGKcrebpA (31) using the Lipofectamine™ 2000 (Invitrogene). For this purpose, 1×10^5 ROSALUC ES cells seeded on gelatinized six-well dishes were co-transfected with 3 μg of circular pPGKcrebpA and 1 μg of circular pSBC2neo (for conferring G418 resistance) along with 10 μl of the Lipofectamine reagent as per the manufacturer's instructions. Treatment and selection of the transfected cells was then performed as described earlier.

Luciferase detection

Cells were harvested from six-well plates and the cell pellet was re-suspended in 50 μl Tris–HCl (pH 7.6). The cell suspension was subjected to repeated freeze-and-thaw cycles (4×) in liquid nitrogen and a 37°C water bath, respectively. After centrifugation (15 000 rpm, 20 min, 4°C) the protein supernatant was used for the luciferase and BCA assays. To detect luciferase activity in the different mouse tissues, the mouse was sacrificed by cervical dislocation and the chosen organs were isolated and frozen in liquid nitrogen. For preparation of lysates, the frozen organs were quickly wrapped in alu-foil and crushed in liquid nitrogen using a chilled mortar and pestle. The powdered tissue was then immediately transferred to a douncer, followed by addition of 400 μl Tris–HCl (pH 7.6) and further homogenized. The homogenized tissue was then subjected to the freeze-thaw cycles and subsequently protein lysates were obtained as described earlier.

An amount of 10μl of the protein lysate was then added to 400 μl of reaction buffer (1:5 ATP solution of 5mM ATP in ddH_2O, pH 7.5, luciferase buffer containing 25 mM glycylglycine, 15 mM $MgSO_4$ in ddH_2O, pH 7.8) in a suitable tube and emitted light was measured with a Lumat LB9507 (Berthold) Luminometer after automatic injection of 50 μl luciferin solution containing 0.1 mM synthetic D-luciferin (Promega), 25 mM glycylglycine in ddH_2O, pH 7.8 (measurement period: 10 s). Luciferase activity was measured in relative light units (RLU). The RLU were normalized to total amount of proteins present in the cell lysate using the BCA assay (32). Moreover, in case of quantitative luciferase detection in the individual organs, luciferase activity of >15 RLU/μg of total protein was considered as real expression.

Standard luciferase assay for absolute determination of luciferase molecules per cell

In order to correlate the luciferase activity to the number of luciferase molecules/cell a luciferase standard using the QuantiLum Luciferase enzyme (Promega) was performed. This QuantiLum Luciferase enzyme was used to generate a Standard Curve. The kinetics of QuantiLum Luciferase and the luciferase gene in ROSALUC are the same and therefore on a theoretical basis, it could be used to estimate the amount of luciferase molecules cell lysates.

The QuantiLum Luciferase enzyme was serially diluted in 1× luciferase buffer (containing 1 mg/ml BSA) to known quantities and corresponding RLU were measured to generate a Standard Curve (duplicates were set up for each serial dilution). This standard curve was then used to further calculate the molecules of luciferase present in the given cell lysates.

Transgenic mice

Transgenic mice were generated by blastocyst injection. *ROSAConL* mice were obtained from *ROSALUC* male mice upon breeding to *K14Cre* female (33) in which Cre is constitutively expressed in oocytes and keratinocytes. Cre deficient mice which show permanent reversion of the luciferase were identified and backcrossed to *Balb/c* to establish *ROSAConL* line.

All mice were bred and kept under standard pathogen free conditions in the animal facility at the Helmholtz Centre for Infection Research, Braunschweig (HZI). Animal experiments were conducted either at HZI or at the University of Ulm according to the guidelines of the German Animal Welfare Law.

Tamoxifen administration to the mice. Mice were orally administered with Tamoxifen by gavage using a special feeding needle (Heiland Vet Gmbh). Tamoxifen tablets (RatioPharm, 30 mg/ml) were dissolved in Clinoleic infusion solution (conc of 20 mg/ml). An amount of 5–8 mg of Tamoxifen was administered orally for 4 days with feeding every alternate day. Mice were sacrificed 5–7 days after the last feed.

In vivo bioluminescence imaging using the Xenogen IVIS 200. Mice expressing the reporter gene, luciferase, were analyzed using the Xenogen IVIS 200 imaging system. For analyzing mice using this imaging technology, the mice were first anaesthetized in a special induction chamber with 2–2.5% isoflurane (Abbot). Upon

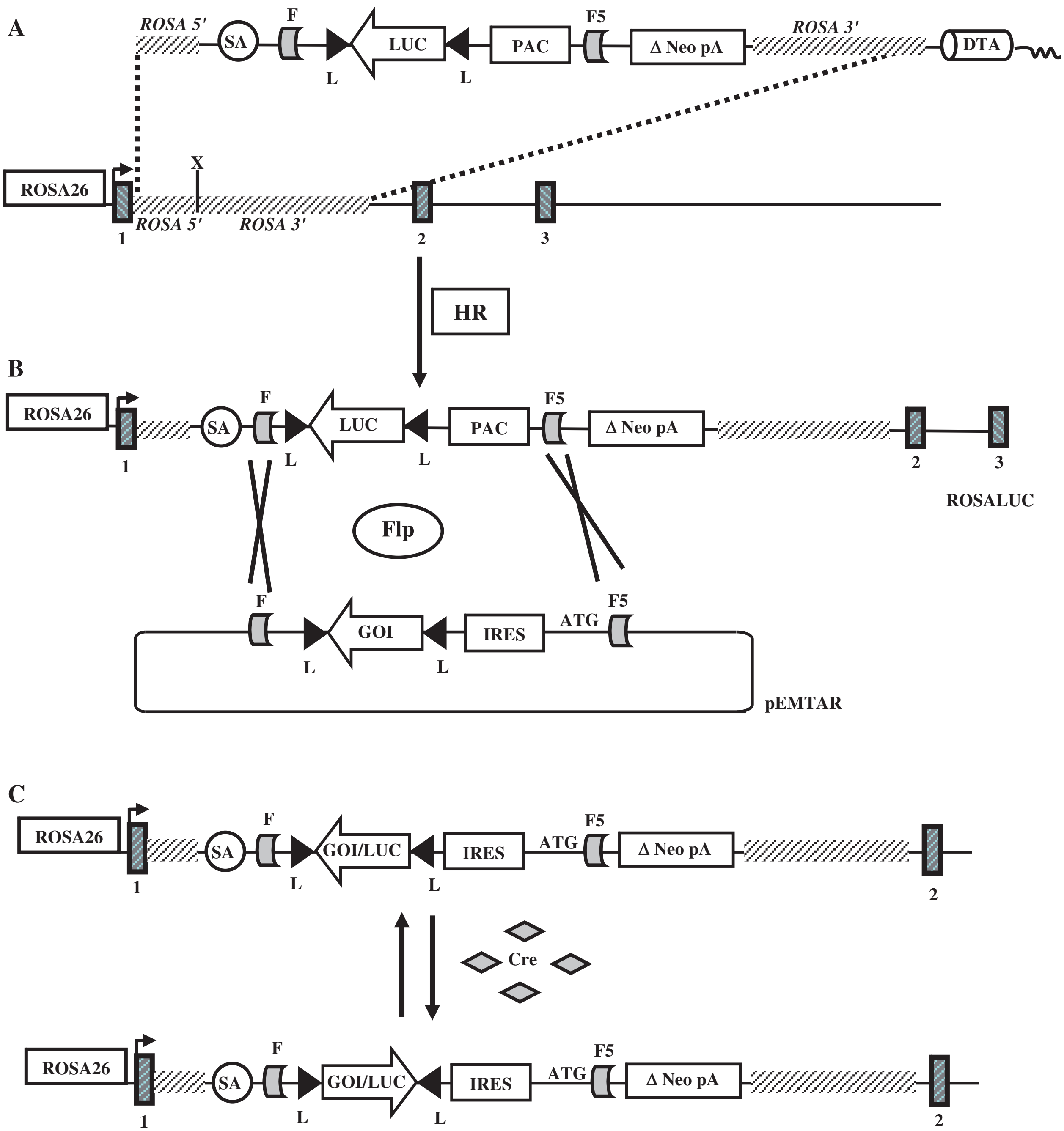


Figure 1. Strategy to create a platform ES cell line for RMCE-based ROSA26 targeting. (**A**) Strategy to make ubiquitously expressed ROSA26 locus RMCE accessible. Structure of the wild-type ROSA26 locus, the tagging vector harboring the heterospecific *FRT* sites and the targeted RMCE compatible locus after homologous recombination (HR) is depicted in the above figure. SA, splice acceptor site; F, wild-type *FRT* site; F5, mutant F5 site; Δneo pA; start-codon deficient neomycin phosphotransferase gene with polyadenylation signal; Rosa5′/3′, ROSA26 genomic flanking sequences; PAC, puromycin N-acetyltransferase gene; LUC, luciferase; L, wild-type *loxP* sites (inversely oriented); HR, homologous recombination; X, *XbaI* restriction site; DTA, Diphtheria toxin A gene. Shaded boxes indicate the exons. (**B**) Targeted integration of expression cassettes of choice into RMCE compatible ES cells via Flp-mediated cassette exchange. The above figure depicts the 'tag and target' strategy to integrate different expression cassettes of choice in the ROSA26 chromosomal background. In the RMCE permissible ROSA26 locus, the two non-interacting *FRT* sites flank the entire expression cassette followed by a 5′-truncated, ATG start codon defective neomycin phosphotransferase gene. The tagged parental ES cells are G418 sensitive. Co-transfection with the Flp recombinase expression plasmid and the targeting vector harboring the corresponding identical heterotypic *FRT* sites will lead to site-directed recombination via F and F5 as indicated by the crosses. After recombination, the defective Δneo gene is complemented by the IRES element and the ATG start codon positioned in-frame thereby rendering the cells undergoing the correct exchange event G418 resistant. The gene of interest (for example the ovalbumin antigen) is also inversely oriented flanked by oppositely oriented *loxP* sites. GOI, gene of interest; Flp, Flp recombinase; RMCE, recombinase mediated cassette exchange; L, wild-type *loxP* site (inversely oriented); IRES, encephalomyocarditis IRES. (**C**) Activation of the floxed GOI/LUC in presence of Cre. Here the gene of interest (GOI) was placed in the reverse orientation with respect to ROSA26 transcription and flanked by *loxP* sites oppositely oriented to each other. Hence this makes the GOI Cre activatable.

intra-peritoneal (i.p) injection with 100µl of luciferin (30mg/ml in PBS, Synchem OHG) the mice were placed in the acquisition chamber equipped with a charge coupled device (CCD) imaging camera. All the images acquired were analyzed using the Living Image 2.60.1 (Igor. Pro 4.09A) software.

Isolation of hepatocytes and coculture with OT-I CD8 T cells

Hepatocytes were isolated as described earlier (34). In brief, the liver was perfused and digested, removed and gently pressed through a mesh. The parenchymal cells were separated from the non-parenchymal cells by centrifugation (500 rpm, 5 min). $CD8^+$ T cells were purified from spleen of TCR transgenic OT-I B6 mice using the $CD8^+$ T-cell MACS isolation kit (catalogue No. 130-090-859; Miltenyi Biotec). A total of 1×10^5 purified $CD8^+$ T cells were cocultured in 200 µl flat-bottom microwells with 1×10^4 hepatocytes. Supernatants were collected from these cocultures at the indicated time-point. IFN-γ were detected in the supernatants by conventional enzyme linked immunosorbent assay (ELISA) as described earlier (34).

Detection of alanine aminotransferase activity

Blood from the retro-orbital sinus of mice was collected in tubes containing anticoagulant (Heparin). The tubes were centrifuged (10 000rpm, 10min) and resulting plasma was used for detecting alanine aminotransferase (ALT) activity. ALT activity was determined using the Reflotron® test (cat.no.745138; Roche, Mannheim, Germany).

Histology

Thin slices of liver tissue (<4mm) were fixed in 4% formalin (pH 7.4) for 24 h and subsequently embedded in paraffin. Paraffin sections, 3µm thick were stained with hematoxylin and eosin (H&E).

RESULTS AND DISCUSSION

Strategy to obtain strictly controlled expression of gene of interest

We constructed a Cre dependent cassette for regulated luciferase expression. This cassette was targeted into the ROSA26 locus by homologous recombination (ROSALUC in Figure 1A). To facilitate re-engineering of this locus in ES cells, the cassette was flanked with *FRT* sites and a non-functional neomycin resistance gene according to a strategy previously shown to be highly efficient for various cell lines (28,35,36). Flp mediated targeting of *FRT* tagged loci is achieved upon transfection with vectors carrying corresponding *FRT* sites and a cassette that activates the neomycin resistance gene according to Figure 1B and C. To evaluate the efficiency of RMCE in these cells various expression cassettes encoding different transgenes and promoters were introduced into the targeting vector pEMTAR (28) and employed for RMCE. As shown in Figure 2, targeting of the ROSALUC ES cells proved to be efficient and highly specific with all tested vectors. Three of the targeted cells were employed to establish transgenic mice and proved to be germ-line competent. Together, ROSALUC cells represent a platform that allows subsequent efficient exchange for cassettes and transgenes of interest and rapid generation of transgenic mice.

In ROSALUC, the luciferase gene was placed in the reverse orientation with respect to ROSA26 transcription and flanked by *loxP* sites oppositely oriented to each other. Cell lysates obtained from targeted ROSALUC mES cells as well as from *in vitro* differentiated cell populations generated thereof were tested for basal luciferase expression. As shown in Table 1, without Cre, luciferase expression of 68 and 23 RLU/µg of total protein was observed in ROSALUC for the ES cells and the *in vitro* differentiated cells, respectively. A value of 20 RLU/µg of total protein corresponds to about three molecules of luciferase per cell (data not shown). At the same time, the wild-type ES cell negative controls showed values ranging from 1 to 7 RLU/µg of total protein which is considered as experimental background. For the following evaluations, we considered luciferase expression levels >15 RLU/µg of total protein as real expression.

The activation of the reporter gene by Cre mediated inversion (Figure 1C) was evaluated upon stable transfection of Cre recombinase. Luciferase activation was monitored before and after *in vitro* differentiation. As shown in Table 1, an ~600-fold induction in luciferase expression was observed before differentiation and a 400-fold induction seen after differentiation in the presence of Cre. This indicates that the luciferase gene in ROSALUC is under strict control of the recombinase and hence activatable. The ROSALUC mES cell clone was subsequently used to establish a transgenic mouse line.

In vivo activation of Cre-dependent luciferase expression in *ROSALUC* transgenic mice

To investigate the control of Cre-mediated activation *in vivo, ROSALUC* transgenic mice were established and mated to the conditional Cre deleter mouse strain, *ROSA26-CreER*^{T2} (37). In *ROSA26-CreER*^{T2} mice, the CreER^{T2} fusion gene is under the control of the ROSA26 promoter and hence ubiquitously expressed in all organs. However, only the presence of the synthetic ligand, Tam, leads to its activation.

ROSALUC mice were mated to *ROSA26-CreER*^{T2} mice and the resulting bitransgenic progeny was analyzed for Tamoxifen (Tam) inducible activation of the luciferase gene by non-invasive bioluminescence imaging (BLI).

Luciferase expression was undetectable in bitransgenic mice in the absence of the inducer (Figure 3A-a). Similarly, lack of bioluminescence was confirmed for the two single transgenic controls, i.e. the *ROSA26-CreER*^{T2} and *ROSALUC* mice, respectively. Importantly, luminescence was undetectable even when applying an exposure time of 5min. This suggests that in *ROSA26-CreER*^{T2}, the activity of the CreER^{T2} fusion is strictly regulated by the inducer and does not show detectable background recombination in the absence of Tam. To study the *in vivo*

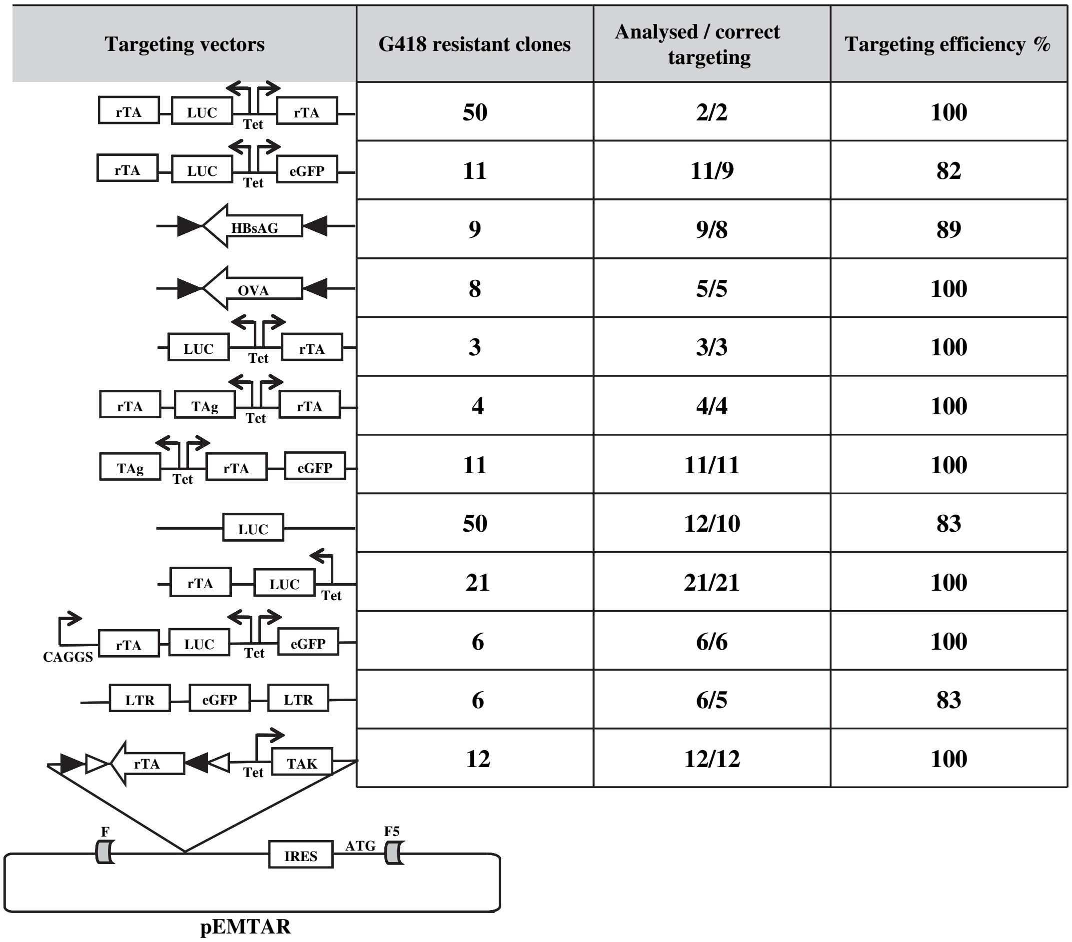


Figure 2. Targeted integration of different antigen/gene cassettes into the parental *FRT* tagged ROSALUC mES cells via RMCE. The above figure gives a summary of the efficiency of integrating different targeting constructs into the tagged ROSA26 locus by Flp-mediated cassette exchange. Different expression cassettes were cloned into the pEMTAR backbone vector (28) harboring the heterotypic *FRT* sites along with the IRES element and the ATG start codon. These targeting vectors were used for subsequent cassette exchange in the RMCE compatible ROSA26 locus. Correct targeting was proven by PCR and/or Southern blot. LUC, luciferase; rTA, reverse tetracycline dependent transactivator; Tet, tetracycline dependent promoter; eGFP, enhanced green fluorescent protein; HBsAg, Hepatitis B surface antigen; OVA, ovalbumin; TAg, SV40 large T antigen; CAGGS, chicken β-actin promoter with cytomegalovirus enhancer; LTR, long terminal repeat; TAK, TAK protein; F, wild-type *FRT* site; F5, mutant F5 site; filled arrow head, wild-type *loxP* site; open arrow head, mutant *loxL3* site.

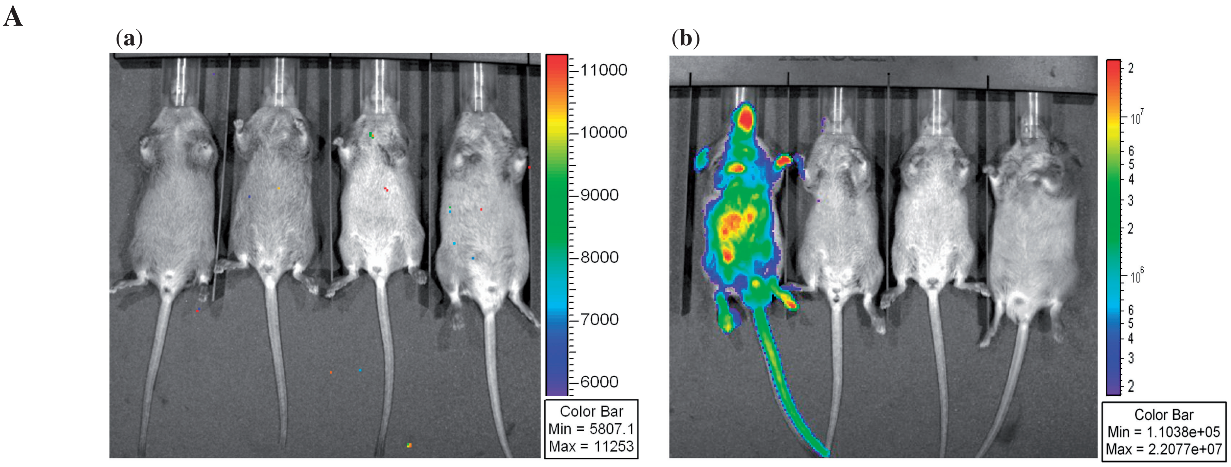
Table 1. Evaluation of luciferase expression in Cre-activatable ROSALUC before and after *in vitro* differentiation of ES cells

	Cre	Luc	RLU/μg of total protein[a]	Fold induction
IB10 ES cell state	–	–	1	N/A
	+	–	2	
IB10 differentiated[b]	–	–	1	N/A
	+	–	7 ± 4	
ROSALUC ES cell state	–	+	68 ± 24	612
	+	+	41 609 ± 3434	
ROSALUC differentiated[b]	–	+	23 ± 5	433
	+	+	9955 ± 510	

$P < 0.001$ (Student's *t*-test comparing values in presence and absence of Cre in the ES cell state as well as after *in vitro* differentiation, respectively).
[a]Mean values from five individual experiments along with the standard deviation are shown in the above table.
[b]Differentiated, after *in vitro* differentiation.
N/A, not applicable.

activation, the mice were induced with Tam. As shown in Figure 3A-b, ubiquitous luciferase expression was detected in the induced double transgenic mouse whereas no background luciferase expression was observed in the non-induced mice. Similarly, no signal was detected in the *ROSA26-CreER*^{T2} and *ROSALUC* single transgenic control mice. These results show that upon Tam induction, the ubiquitously expressed CreER^{T2} fusion protein mediates recombination between the inversely oriented *loxP* sites, resulting in the activation of luciferase. Also, no detectable luciferase expression in the single transgenic *ROSALUC* control mouse by BLI further proves that in the absence of Cre recombinase, there is no background luciferase expression.

For quantitative evaluation, bitransgenic *ROSALUC X ROSA26-CreER*^{T2} mice (induced and non-induced) were sacrificed. Luciferase was assayed in various tissue samples. As shown in Figure 3B, ubiquitous luciferase



Luc	+	+	-	+
CreER^{T2}	+	+	+	-
Tam	-	-	-	-

Luc	+	+	-	+
CreER^{T2}	+	+	+	-
Tam	+	-	+	+

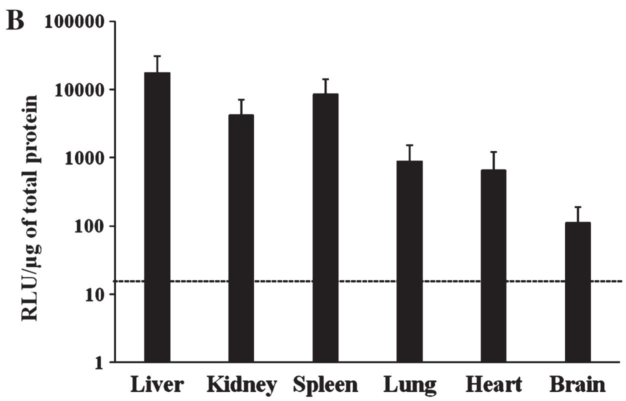


Figure 3. *In vivo* activation of Cre-dependent luciferase expression in bitransgenic *ROSALUC X ROSA26-CreER*^{T2} mice. (**A**) *In vivo* non-invasive bioluminescent imaging (BLI) of *ROSALUC X ROSA26-CreER*^{T2} offsprings. (a) BLI image of non-induced animals. Four-weeks-old bitransgenic *ROSALUC X ROSA26-CreER*^{T2} mice along with single transgenic *ROSA26CreER*^{T2} and *ROSALUC* as controls are indicated. (b) BLI image of animals after Tam induction. Image was acquired 5 days after the last Tam feed. Color bar indicates photons/cm^2/s/steradian with the minimum and maximum threshold values. (**B**) Monitoring luciferase expression in the different organs isolated from double transgenic *ROSALUC X ROSA26-CreERT*^{2} mice. The 4–8-weeks-old bitransgenic *ROSALUC X ROSA26-CreERT*^{2} mice (induced and uninduced) were sacrificed and various organs were isolated. Tissue lysates obtained were subjected to a luciferase assay. The luciferase activity observed in RLU was normalized to micrograms of total protein present in the tissue sample. Figure depicts induced *ROSALUC X ROSA26-CreERT*^{2}. Non-induced *ROSALUC X ROSA26-CreERT*^{2} as well as the control single transgenic *ROSALUC and ROSA26-CreERT*^{2} mice showed an average of <2 RLU/µg of total protein and are not depicted in the figure. For each group four mice were analyzed.

expression was detected in all investigated tissue samples obtained from induced *ROSALUC X ROSA26-CreER*^{T2} mice. The values obtained for the non-induced bitransgenic mice were <2 RLU/µg which is comparable to the values obtained from the single transgenic *ROSALUC* and *ROSA26-CreER*^{T2} control mice (not shown). These quantitative luciferase data thus confirm the observations made by non-invasive BLI.

The fact that Cre induced expression is detected in brain is different from previous studies employing the *lacZ* reporter, where no expression in brain could be detected upon Tam administration (37). This indicates that the luciferase reporter is more sensitive and can monitor even a low Cre activity in brain, which is probably impaired due to the inefficient transfer of Tam across the blood–brain barrier. Such a limitation might be overcome in optimized feeding protocols.

As a control, we evaluated the luciferase expression in *ROSAConL* mice which constitutively express luciferase from the ROSA26 promoter. *ROSAConL* were obtained from *ROSALUC* upon mating to *K14Cre* mice (33) and screening the progeny for permanent inversion of the luciferase cassette and absence of Cre. As shown in Figure 4, generally, more homogenous expression of luciferase was detected. Also, lower levels of luciferase were monitored in *ROSAConL* mice if compared to

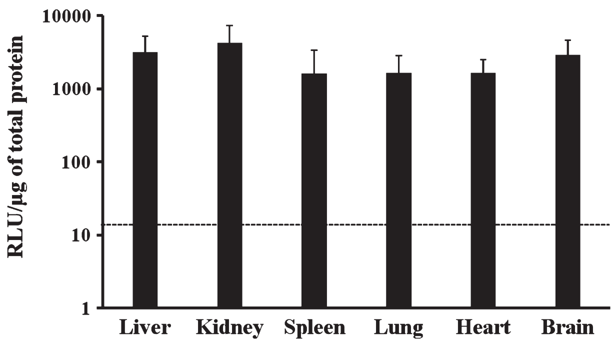


Figure 4. Monitoring luciferase expression in the different organs isolated from *ROSAConL* mice. The 4–8-weeks-old *ROSAConL* mice were sacrificed and various organs were isolated. Tissue lysates obtained were subjected to a luciferase assay. The luciferase activity observed in RLU was normalized to micrograms of total protein present in the tissue sample. Number of mice analyzed = 6.

ROSALUC X ROSA26-CreER^{T2} as shown in Figure 3. We attribute strain specific properties for the various levels of ROSA26 activity in the two mice since the *ROSAConL* mice have been backcrossed to *Balb/C* to higher generations, while the *ROSALUC X ROSA26-CreER*^{T2} animals display a mixed background of *129/OLA, Balb/C* and *C57/Bl6*. The impact of the genetic background on expression of (*trans*-)genes and promoter activity has been observed in other studies (38–40).

We also evaluated Cre mediated activation of luciferase mRNA by RT–PCR. Lung and liver were used for this purpose. As shown in the Supplementary Data, inversion of the luciferase cassette was observed for the Tam treated double transgenic animals as well as for *ROSAConL* control mice, while tissues from non-induced mice did not show any band after 30 cycles of amplification. Thus, the RT–PCR results confirm tight regulation of the cassettes.

Together, the results obtained by BLI, quantitative luciferase expression and RT–PCR indicate a strict Tam-inducible Cre-mediated activation of the luciferase reporter gene. Moreover, the data provided in Figure 3A and B clearly exclude any Cre-independent activation of the cassette (e.g. due to chromosomal read-in from 3′-promoter (41) and expression of luciferase from an antisense transcript). Thus, the design of the Cre reporter construct as shown in Figure 1 allows for tight regulation.

Tight control in *ROSALUC* mice expressing high levels of CreER^{T2}

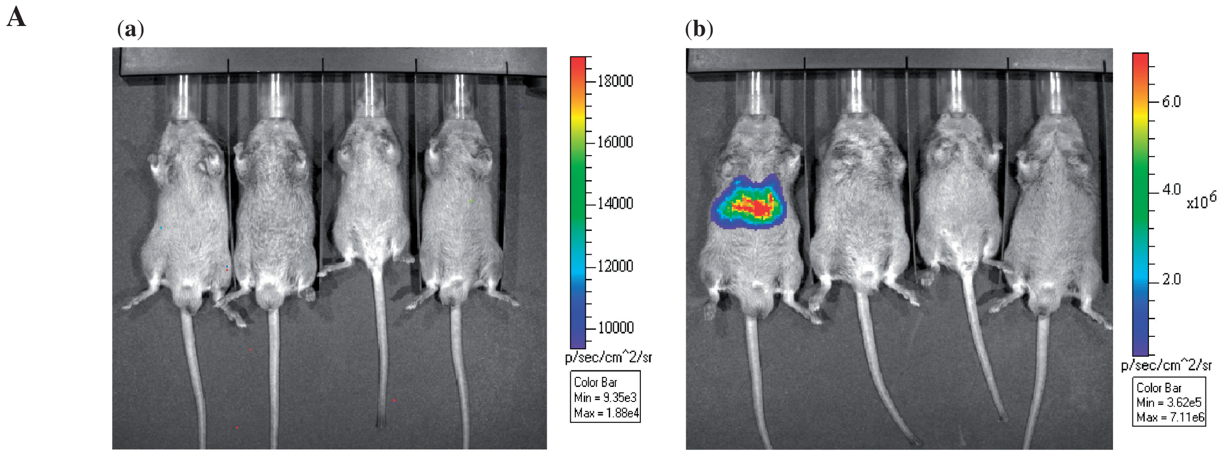
The absence of expression in *ROSALUC* single transgenic mice clearly excludes any leakiness due to the reporter cassette *per se*. However, accidental activation of the transgene cassette could also occur due to leakiness in the Cre control, in fusion proteins of Cre and the hormone receptor moieties (42–44). It is discussed that proteolytic cleavage of the fusion protein is the molecular cause of this (22,37,42,44). Alternatively, CreER^{T2} might enter the nucleus upon cell division. For both mechanisms the expression level of CreERT^{2} would affect basal activity and thus leakiness. Indeed, Imayoshi *et al.* (42) demonstrated that the expression level of CreER^{T2} is a crucial factor for obtaining Tam-mediated regulation. Thus, the tight control of our system as depicted in Figure 3 might be associated to limited CreER^{T2} expression as a consequence of the moderate expression level mediated by the ROSA26 promoter.

We tested if our system would still confer strict regulation in presence of high level expression of CreER^{T2}. For this purpose *ROSALUC* was crossed to *Alb-CreER*^{T2} mice in which the CreER^{T2} coding sequence is inserted into the serum albumin locus (45). *Alb-CreER*^{T2} mice were shown to selectively express CreER^{T2} in almost all hepatocytes in the adult liver (45) and activate Cre upon Tam administration. Moreover, the albumin promoter is highly expressed in hepatocytes (46).

In a similar approach as described earlier, double transgenic *ROSALUC X Alb-CreER*^{T2} mice were imaged for bioluminescence in the non-induced state (Figure 5A-a). No bioluminescent signal was detected in the liver or any other tissue. To investigate the liver-specific activation of the floxed luciferase gene *in vivo*, the mice were induced with Tam. As it can be seen in Figure 5A-b, activation of the luciferase gene was detected in the central area of the induced bitransgenic mouse. Quantitative luciferase data were also obtained from the different organs. As seen in Figure 5B, in the absence of Tam a residual luciferase expression level of ~20 RLU/µg of total protein was observed in the livers of the double transgenic mice in contrast to the single transgenic control mice. This activity corresponds to approximately three luciferase molecules per cell. Interestingly, a residual expression level could not be detected by RT–PCR (Supplementary Data). All other organs isolated from these non-induced bitransgenic mice did not show any background luciferase expression (data not shown). A dramatic (244-fold) activation in luciferase expression was seen selectively in the liver of the induced double transgenic mice when compared to the expression data obtained for the non-induced mice. This amount of luciferase expression in the liver corresponds to approximately 700 luciferase molecules per cell. Also, no luciferase expression was observed for the single transgenic control mice. RT–PCR confirmed the tight regulation in this model (Supplementary Data).

Previously, it was shown that efficiency of Cre mediated recombination is affected by the nature and accessibility of the chromosomal site of the recombination targets (37,44,47). Here, we show that apart from this, a high level of CreER^{T2} expression in a specific tissue can account for leaky expression. The moderate expression level of CreER^{T2} from the ROSA26 promoter does not induce any background expression, but is sufficient to activate the target gene. Expression from other regulatory elements might differ and thus, lead to elevated background levels of the target gene, as it is the case for the albumin promoter driven CreER^{T2}.

Together, these data highlight the requirement for careful evaluation of a specific combination of CreER^{T2}



Luc	+	+	-	+
CreER^{T2}	+	+	+	-
Tam	-	-	-	-

Luc	+	+	-	+
CreER^{T2}	+	+	+	-
Tam	+	-	+	+

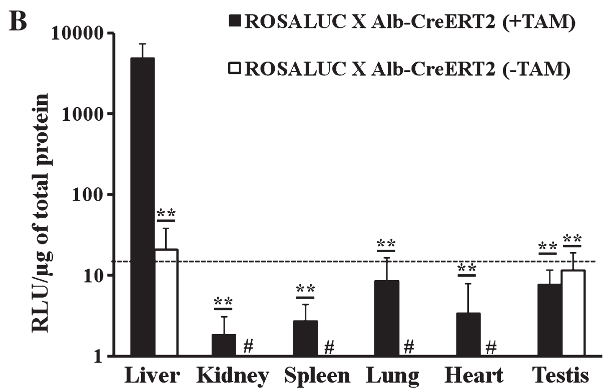


Figure 5. *In vivo* activation of Cre-dependent luciferase expression in bitransgenic *ROSALUC X Alb-CreER^{T2}*mice. (**A**) *In vivo* non-invasive bioluminescent imaging (BLI) of *ROSALUC X Alb-CreER^{T2}* mice offsprings. (a) BLI image of animals not induced with Tam. Four-weeks-old bitransgenic *ROSALUC X Alb-CreER^{T2}* mice along with single transgenic *Alb-CreER^{T2}*and *ROSALUC* as controls are indicated. (b) BLI image of animals induced with Tam. Image was acquired 5 days after the last Tam feed. Color bar indicates photons/cm^2/s/steradian with the minimum and maximum threshold value. (**B**) Monitoring luciferase expression in the different organs isolated from *ROSALUC X Alb-CreERT^2* mice. The 4–8-weeks-old bitransgenic *ROSALUC X Alb-CreERT^2* mice (induced and uninduced) were sacrificed and various organs were isolated. Tissue lysates obtained were subjected to a luciferase assay. Figure depicts induced and non-induced *ROSALUC X Alb-CreERT^2*. Hash sign indicates values <1 RLU/µg of total protein. Brain tissue sample from induced and non-induced mice showed values <1 RLU/µg of total protein and is not shown in the figure. Values above dashed line are considered as luciferase expression. Tissues from control single transgenic *ROSALUC* and *Alb-CreERT^2* mice showed an average of <2 RLU/ µg of total protein and are not depicted in the figure. Number of mice analyzed for each group = 5. Student's *t*-test, comparing values to induced liver results in $^{**}P < 0.01$.

effector expression and the position of the *loxP* reporter system to validate the performance. In this respect, more advanced fusions of Cre with the estrogen receptor moieties might help to overcome limitations due to Cre mediated leakiness (23,48).

Immunological assay to evaluate the tightness of gene regulation

The results obtained from the luciferase reporter mice confirmed tight regulation of the transgene. Still, a low basal luciferase activity was detected in the liver. This residual activity however, was accompanied with a high statistical variation. If this basal activity is due to intrinsic fluctuation of expression in the mice or to experimental errors is not clear.

The immune response to antigens is an extremely sensitive *in vivo* assay that monitors any accidental activation by rendering the animals tolerant towards the respective antigen. We and others have shown that when low expressed protein are not detectable using sensitive biochemical methods, these little expression levels could provide a strong immune response (49). We decided to make use of this highly sensitive biological activity to challenge the tightness of the above described system. To test the strictness of gene regulation we integrated the Ovalbumin gene (OVA) as a model antigen into the ROSA26 locus via RMCE. The OVA antigen was flanked with inverse *loxP* sites and placed in antisense orientation according to the reporter gene configuration (Figure 1). ROSAOVA subclones obtained upon correct exchange event were confirmed by Southern blot as well as PCR analysis (data not shown). A transgenic mouse line was established and subsequently mated to the *Alb-CreER*^{T2} mice (45) to obtain double transgenic *ROSAOVA X Alb-CreER*^{T2} progeny.

Cre-activatable OVA expression and presentation of the OVA epitope by MHC-I in hepatocytes isolated from *ROSAOVA X Alb-CreER*^{T2} mice was tested. For this purpose, an *in vitro* coculture assay was performed (Figure 6). Hepatocytes were isolated from six male double transgenic mice of which three were induced with Tam and three were non-induced. The hepatocytes were then cocultured with CD8$^+$ T cells from OT-I T cell receptor transgenic mice for 72 h. OT-I CD8$^+$ T cells express T cell receptor specific for the OVA peptide which would get activated upon recognition of OVA epitope in context with MHC-I. This activation, in turn, can be monitored by IFN-γ release by the T cells. As can be seen in Figure 6, the OT-I CD8$^+$ T cells cocultured with hepatocytes isolated from the three induced mice showed proliferation and activation with IFN-γ release of as high as 1ng/ml whereas for the non-induced mice, no proliferation was observed similar to the single transgenic controls. The result from this experiment confirms the strict Cre-dependent and inducible activation of the transgene-encoded antigen expression in the hepatocytes of *ROSAOVA X Alb-CreER*^{T2} mice. Importantly, when subjecting the non-parenchymal cell fraction from the livers of these mice to this assay, no IFN-γ release was detected, clearly excluding unintended presentation of OVA from these cells (data not shown). This indicates that the OVA antigen expression and presentation of its epitope is strict hepatocyte-specific.

Finally, we tested if upon induction of antigen (OVA) expression in hepatocytes, OVA would be recognized as a newly expressed protein (neo antigen) and thus results in an immune response. For this purpose, we bred the mice to OT-I mice (50). In the resulting triple transgenic *OT-1 X ROSAOVA X Alb-CreER*^{T2} mice, OT-I CD8$^+$ T cells were not deleted and were detected at levels comparable to control OT-I mice mice (data not shown). Moreover, the mice displayed normal levels of ALT, a serum enzyme that is released upon killing of hepatocytes (Figure 7A). This indicates that in the non-induced state the T cells are not activated.

Upon induction of OVA expression by Tam we monitored the ALT. A massive increase of ALT was detected 2 days upon induction (Figure 7A). The mice were sacrificed and liver samples showed infiltration of mononuclear cells and dying hepatocytes (Figure 7B). Together, this shows that the OVA antigen is recognized as a neo antigen and is thus strictly controlled in this model.

Together, we show that the strategy of inverse integration of a floxed transgene into the ubiquitously active ROSA26 locus provides tightly controlled transgene expression. Due to the availability of this locus via

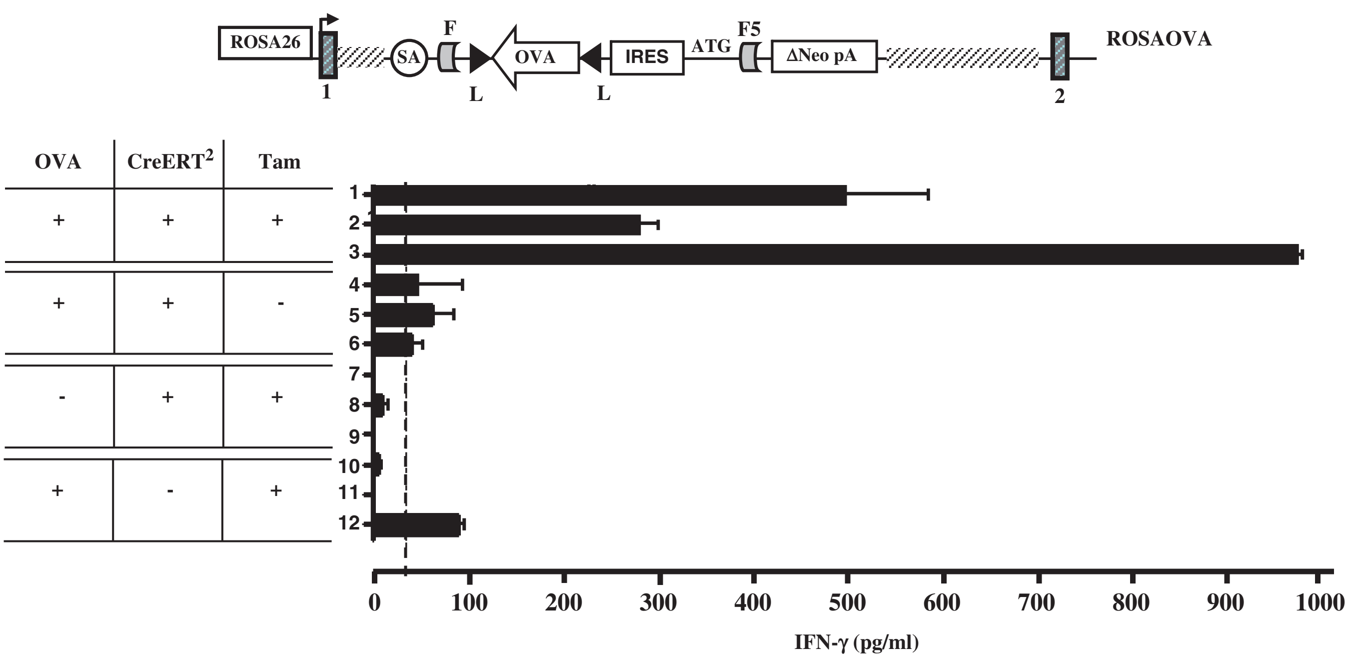


Figure 6. *In vitro* coculture assay to evaluate Cre-activatable OVA expression in hepatocytes from *ROSAOVA X Alb-CreER*^{T2} mice. The above figure depicts the OVA antigen cassette as integrated in the ROSA26 locus in *ROSAOVA* mice via RMCE. *ROSAOVA* mice were mated to *Alb-CreER*^{T2}. Eight-weeks-old double transgenic mice were used to evaluate activation of OVA expression. Hepatocytes were isolated from six male double transgenic *ROSALUC X Alb-CreER*^{T2} mice of which three were induced with Tam (mice Nr 1–3) and three non-induced (mice Nr 4–6); these cells were cocultured with OT-I CD8+ T cells. As negative controls single transgenic *Alb-CreER*^{T2} mice (Nr 7–9) and *ROSAOVA* mice Nr (10–12) mice were used. Activation of OT-I T cells was monitored via IFN-γ cytokine secretion in the supernatants by conventional ELISA.

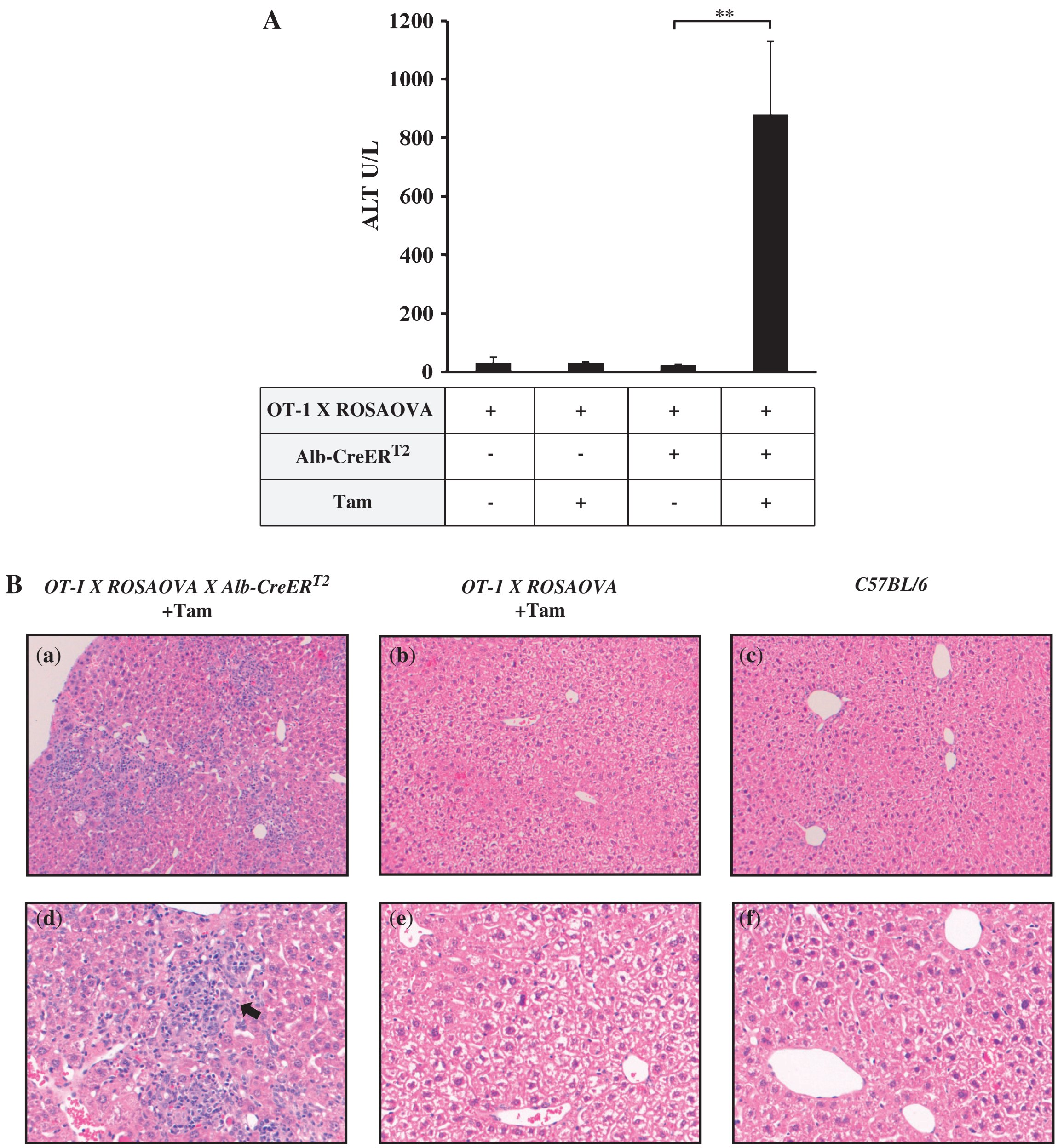


Figure 7. Induced hepatitis upon Tam treatment in *OT-I X ROSAOVA X Alb-CreER^{T2}* mice. (**A**) Determination of ALT activity in blood of *OT-I X ROSAOVA X Alb-CreER^{T2}* mice. The 8-weeks-old *OT-I X ROSAOVA X Alb-CreER^{T2}* were induced with Tam and blood was collected for ALT analysis at Day 2. Number of mice analyzed for each group = 4. $^{**}P < 0.01$ (student's *t*-test). (**B**) Histology of mouse liver tissue. Paraffin embedded liver tissue section was stained with H&E. Liver histology of *OT-I X ROSAOVA X Alb-CreER^{T2}* (a and d), control *OT-I X ROSAOVA* (b and e) and C57BL/6 (c and f) is shown with different magnifications ×100 (a–c) and ×200 (d–f). Arrow indicates mononuclear cell infiltration.

RMCE, this strategy represents a flexible platform for the establishment of transgenic mouse models in which tight regulation of the transgene is crucial.

The strength of target gene activation depends on its accessibility towards inversion and on the strength of its expression. In the experimental design presented here, wild-type *loxP* sites were employed. As a consequence continuous 'flipping' (repeated inversion) will occur as long as Cre recombinase is present. Hence, theoretically only 50% of the cells will express luciferase. For certain applications, however, 100% expressing cells maybe required whereby this system could then be exploited by using mutant *loxP sites* for effecting a permanent switch (51–53). Thus, by choosing appropriate expression conditions for CreER^{T2} and the target gene, maximal gene activation with minimal background/basal expression can be reached.

As immunological assays are exquisitely specific and sensitive (far beyond the resolution of most biochemical assays), the data presented exclude biologically relevant leakiness. We cannot exclude that for some applications such as activation of an oncogene in a tumor initiating cell, stochastic gene activations could lead to consequences even in this experimental setting. The flexibility of the described system however allows for rapid testing of various cassette designs and has hence a unique advantage to facilitate easy access to mouse models that address such questions.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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